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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

Attorney's Docket No. 2577-112

U.S. Application No. (if known, see 37 CFR 1 5) 10/019992

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/SG99/00061	June 22, 1999	

TITLE OF INVENTION
Detection of *Salmonella Enteritidis*

APPLICANT(S) FOR DO/EO/US
Hwei-Sing KWANG, Wei LIU, Su-Shing Sharon LOW, Kwang Yeng Hilda LOH

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has **NOT** expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

ITEMS 11. TO 16. below concern other document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A **FIRST** preliminary amendment.
    [ ] A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information: Courtesy copy of International Application No. PCT/SG99/00061 w/ attached International Search Report.

U.S. APPLICATION NO. (If known, see 37 CFR 1.50) 10/019992	INTERNATIONAL APPLICATION NO PCT/SG99/00061	ATTORNEY DOCKET NO 2577-112

17. [X] The following fees are submitted:		CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492)(a)(1)-(5):			
Search Report has been prepared by the EPO or JPO	$ 890.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482)	$ 710.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))	$ 740.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO	$ 1,040.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	$ 100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =		$ 890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	19 -20 =	0	X $18.00	$	
Independent Claims	3 - 3 =	0	X $84.00	$	
Multiple dependent claim(s) (if applicable)			+ $280.00	$	
TOTAL OF ABOVE CALCULATIONS =				$890.00	
Reduction by 1/2 for filing by small entity, if applicable. Applicant claims small entity status.				$445.00	
SUBTOTAL =				$445.00	
Processing fee of $130.00 for furnishing the English translation later [ ] 20 [ ] 30 than months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$445.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED =				$445.00	
				Amount to be refunded	$
				charged	$

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 02-2135 in the amount of $445.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☐ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-2135. A duplicate copy of this sheet is enclosed.

d. ☐ Payment by credit card. (Form PTO-2038 enclosed.)

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE	*Application Number*	§371 of PCT/SG99/00061
	Filing Date	June 22, 1999
	First Named Inventor	Hwei-Sing KWANG
	Group Art Unit	Unassigned
	Examiner Name	Unassigned
	Attorney Docket Number	2577-112
Title of the Invention: DETECTION OF *SALMONELLA ENTERITIDIS*		

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

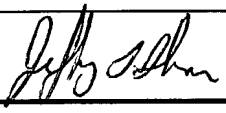
Please amend the above-identified U.S. patent application as follows:

IN THE CLAIMS:

Please cancel claims 20-34.

REMARKS

The above is being made to make amendment to the claims prior to examination on the merits. The amendment does not add to or depart from the original disclosure, or constitute prohibited new matter.

RESPECTFULLY SUBMITTED,					
NAME AND REG. NUMBER	Jeffrey L. Ihnen, Registration No. 28,957				
SIGNATURE			DATE	December 26, 2001	
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City	Washington	*State*	D.C.	*Zip Code*	20004
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Nucleotide sequence of *sefA*

ATTTTGTAATATGCGTAAATCAGCATCTGCAGTAGCAGTTCTTGCTTTAATTGCAT
GTGGCAGTGCCCACGCAGCTGGCTTTGTTGGTAACAAAGCAGAGGTTCAGGCAGC
GGTTACTATTGCAGCTCAGAATACAACATCAGCCAACTGGAGTCAGGATCCTGGC
TTTACAGGGCCTGCTGTTGCTGCTGGTCAGAAAGTTGGTACTCTCAGCATTACTG
CTACTGGTCCACATAACTCAGTATCTATTGCAGGTAAAGGGGCTTCGGTATCTGG
TGGTGTAGCCACTGTCCCGTTCGTTGATGGACAAGGACAGCCTGTTTTCCGTGGG
CGTATTCAGGGAGCCAATATTAATGACCAAGCAAATACTGGAATTGACGGGCTTG
CAGGTTGGCGAGTTGCCAGCTCTCAAGAAACGCTAAATGTCCCTGTCACAACCTT
TGGTAAATCGACCCTGCCAGCAGGTACTTTCACTGCGACCTTCTACGTTCAGCAG
TATCAAAAC

(SEQ ID NO:1)

Amino acid sequence for SEF14:

1

MRKSASAVAVLALIACGSAHAAGFVGNKAEVQAAVTIAAQNTTSANW
SQDPGFTGPAVAAGQKVGTLSITATGPHNSVSIAGKGASVSGGVATVP
FVDGQGQPVFRGRIQGANINDQANTGIDGLAGWRVASSQETLNVPVT
TFGKSTLPAGTFTATFYVQQYQN

165

Amino acid sequence for the C128 fragment of SEF14:

AAQNTTSANWSQDPGFTGPAVAAGQKVGTLSITATGPHNSVSIAGKGA
SVSGGVATVPFVDGQGQPVFRGRIQGANINDQANTGIDGLAGWRVASS
QETLNVPVTTFGKSTLPAGTFTATFYVQQYQN

(SEQ ID NO:3)

Amino Acid Sequence of *S.enteritidis* Flagellin Antigen

LTQNNLNKSQSSLSSAIERLSSGLRINSAKDDAAGQAIANRFTS
NIKGLTQASRNANDGISIAQTTEGALNEINNNLQRVRELSVQATNGTNSDSDLKSIQD
EIQQRLEEIDRVSNQTQFNGVKVLSQDNQMKIQVGANDGETITIDLQKIDVKSLGLDG
FNVNGPKEATVGDLKSSFKNVTGYDTYAAGADKYRVDINSGAVVTDAAAPDKVYVNAA
NGQLTTDDAENNTAVDLFKTTKSTAGTAEAKAIRGAIKGGKEGDTFDYKGVTFTIDTK
TGDDGNGKVSTTINGEKVTLTVADIATGATDVNAATLQSSKNVYTSVVNGQFTFDDKT
KNESAKLSDLEANNAVKGESKITVNGAEYTANATGDKITLAGKTMFIDKTASGVSTLI
NEDAAAAKKSTANPLASIDSALSKVDAVRSSLGAIQNRFDSAITNLGNTVTNLNSARS
RIEDADYATEVSNMSKAQILQQAGTSVLAQANQVPQNVLSLLR
(SEQ ID NO:4)

90 amino acid fragment of *S. enteritidis* flagellin antigen

TAEAKAIRGAIKGGKEGDTFDYKGVTFTIDTKTGDDGNGKVSTTINGEKVTLTVADIA
TGATDVNAATLQSSKNVYTSVVNGQFTFDDKT
(SEQ ID NO:5)

fragment A: 69 amino acids (aa 258-327 of SEQ ID NO:4)

KEGDTFDYKGVTFTIDTKTGDDGNGKVSTTINGEKVTLTVADIATGATDVNAATLQSSKN
VYTSVVNGQ

(SEQ ID NO:6)

fragment B: 40 amino acids (aa 276-316 of SEQ ID NO:4)

KTGDDGNGKVSTTINGEKVTLTVADIATGATDVNAATLQS

(SEQ ID NO:7)

fragment C: 27 amino acids (aa 279-306 of SEQ ID NO:4)

DGNGKVSTTINGEKVTLTVADIATGAT

(SEQ ID NO:8)

fragment D: 11 amino acids (aa 285-296 of SEQ ID NO:4)

STTINGEKVTL

(SEQ ID NO:9)

DETECTION OF *SALMONELLA ENTERITIDIS*

FIELD OF THE INVENTION

This invention relates to a method for detecting *Salmonella enteritidis* in poultry and in their eggs. More specifically, the invention is directed to a method for detecting *S. enteritidis* which comprises contacting a biological sample obtained from poultry suspected of containing *S. enteritidis* with a fragment of the *S. enteritidis* fimbrial protein or a fragment of the *S. enteritidis* flagellin protein which specifically recognizes *S. enteritidis* antibodies present in the sample and discriminates between *S. enteritidis* and other *Salmonella spp.*

BACKGROUND OF THE INVENTION

Salmonella enteritidis, an agent which causes salmonellosis in poultry, can be transmitted vertically from laying hens to eggs. Consumption of eggs or meat contaminated with the organism can lead to food poisoning in humans. This is a worldwide problem in public health; in the U.S. alone, more than a million cases of salmonellosis are reported annually. Outbreaks in the elderly and in young children can be especially dangerous, resulting in severe gastroenteritis and possibly fatal septicemia.

Possible *Salmonella* virulence factors include fimbrial structures, which are gene products involved in the invasion of eukaryotic cells, and lipopolysaccharides. Another factor is the flagella, which confer motility to the bacterium and so contribute to the bacterium's colonization.

In view of the large number of cases of *Salmonella enteritidis* cases reported each year, there is an obvious need for a reliable method for detecting laying flocks infected with *S. enteritidis*. Bacteriological techniques for the isolation of *S. enteritidis*, such as those disclosed by Williams, J.E., and A.D. Whittemore, *Avian Disease*, 20:728 (1976), are laborious, time-consuming and costly. False-negative results can arise when *S. enteritidis* is overgrown by other *Salmonella* serotypes present in the samples. In addition, these methods may not identify all birds infected with *S. enteritidis* because *Salmonella* excretion is intermittent.

There are some existing serological methods, such as the serum plate test (SPT), latex agglutination test (LAT) and enzyme linked immunosorbent assay (ELISA), which are rapid and easy to perform, and antibodies against *S. enteritidis* have been found at relatively high levels in the sera of infected chickens, making antibody detection practical. Most ELISAs utilize either lipopolysaccharides (LPS) or flagella antigens for the detection of antibody against *S. enteritidis*. The use of these antigens, however, has resulted in false positive results in recent reports, making discrimination between *Salmonella* serotypes difficult.

See Christopher, J., et al. *J. Clin. Microbiol.* 34:792-797 (1996); van Zijderveld, Fred, *J. Clin. Microbiol.* 36:2560-2566 (1992); Barrow, P.A., *Epidemiology and Infection* 109:361-369(1992); and Barrow, P.A., *Int. J. Food Microbiol.* 21:55-68 (1994). Accordingly, further methods are sought.

SUMMARY OF THE INVENTION

This invention is directed to a method for detecting *S. enteritidis* which comprises contacting a biological sample obtained from poultry suspected of being infected with *S. enteritidis* with an antigenic fragment of an *S. enteritidis* fimbrial protein or an antigenic fragment of an *S. enteritidis* flagellin protein, which fragment specifically recognizes antibodies against *S. enteritidis* present in the sample and discriminates between antibodies against *S. enteritidis* and antibodies against other *Salmonella spp*, under conditions sufficient for the formation of an immunological complex between *S. enteritidis* antibodies present in the sample and the antigenic fragment and then detecting the formation of such a complex.

Another embodiment of this invention comprises a method for detecting *S. enteritidis* which comprises contacting a biological sample obtained from poultry suspected of being infected with *S. enteritidis* with a combination of an antigenic fragment of an *S. enteritidis* fimbrial protein and an antigenic fragment of an *S. enteritidis* flagellin protein, each of which fragments specifically recognizes antibodies against *S.*

enteritidis present in the sample and discriminates between antibodies against *S. enteritidis* and antibodies against other *Salmonella spp*, under conditions sufficient for the formation of an immunological complex between *S. enteritidis* antibodies present in the sample and either or both of the antigenic fragments and then detecting the formation of such a complex.

This invention further is directed to a diagnostic kit, which comprises an antigenic fragment of the *S. enteritidis* fimbrial protein, an antigenic fragment of the *S. enteritidis* flagella protein, or both such fragments, wherein said fragment(s), when combined with a biological sample obtained from poultry suspected of being infected with *S. enteritidis*, specifically recognizes antibodies against *S. enteritidis* present in said sample and discriminates between antibodies against *S. enteritidis* and antibodies against other *Salmonella spp*.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 sets forth the DNA sequence encoding the *S. enteritidis* fimbrial protein SEF14.

Figure 2A sets forth the amino acid sequence of the *S. enteritidis* fimbrial protein SEF14 and Figure 2B sets forth the amino acid sequence of the fragment identified herein as C128.

Figure 3 is a chart illustrating eight subfragments of fimbrial protein SEF14 that were tested for reactivity to *S. enteritidis*.

Figure 4 is a representation of the full-length *S. enteritidis* flagellin antigen and the fragments obtained from the full-length sequence as described in Example 2.

Figure 5 sets forth the amino acid sequence of the full length *S. enteritidis* flagellin protein illustrated in Figure 4, as well as the amino acid sequence encoded by the 270 bp fragment and the amino acid sequence of each of four subfragments which were isolated as described in Example 2.

DETAILED DESCRIPTION OF THE INVENTION

Determination of antigenic fragments

A. Antigenic fragments of fimbrial antigen

A fimbrial antigen designated SEF14 was first described in 1994 (Thorns, C.J., et al., *J. Clin. Microbiol.* 28:2409-2414). The SEF14-encoding gene, designated *sefA*, was shown to be limited in distribution to serotypes belonging to *Salmonella* serogroup D. Expression of the SEF14 antigen as a surface structure has been detected only in *S. enteritidis, S. dublin, S. blegdam* and *S. moscow*, but *S. enteritidis* is the only serotype that can be isolated from poultry, and SEF14 fimbriae is expressed by all *S. enteritidis* strains. It is known that antibody against SEF14 is developed following infections with *S. enteritidis*. The entire sequence of the *sefA* gene is known and has been published in the literature (Thorns, C.J., et al., *J. Clin. Microbiol.* 4(34):792-797 (1996)) and in GenBank (accession number L03833). The DNA sequence of the *sefA* gene is provided

in Figure 1 and is identified herein as SEQ ID NO. 1. The amino acid sequence for SEF14 is provided in Figure 2A. This sequence is identified herein as SEQ ID NO. 2.

To determine which portions of SEF14 specifically recognize *S. enteritidis*, the *sefA* gene was amplified from genomic DNA with primers designated from Genbank sequence data. The resulting amplified fragment was cloned into an expression vector. The vector was chosen such that the *sefA* gene product would be expressed as a fusion protein. A preferred fusion partner is glutathione -S- transferase of *Schistosoma japonica* (GST).

The reactivity of the resultant recombinant GST-SEF14 fusion protein was tested with sera from chickens experimentally infected with *S. enteritidis* and other Salmonella serotypes in immunoblot. GST-SEF14 was recognized by all *S. enteritidis*-infected sera, and weak reaction bands also were obtained with sera from infection of *S. senftenberg, S. amsterdam, S java, S. pullorum, S. typhimurium, S. agona* and *S. oranienberg*. No reactions were found with *S. haardt, S. montevideo, S. paratyphimurium, S. emek* and pre-infection sera. From these data it appeared that the SEF14 fusion protein shares some common epitopes with other *Salmonella* serotypes which caused these cross-reactions.

In order to locate a partial SEF14 fragment specific to *S. enteritidis*, eight sub-fragments of the 165 amino acid SEF14, as shown in Figure 3, were expressed as fusion proteins with GST. The reactivity

of each fragment was tested by immunoblot assays. Of the three fragments containing the N-terminus of SEF14, only the full-length SEF14, named F165 (165 aa), was recognized by all sera from *S. enteritidis* infections. The other two fragments, N150 (150 aa) and N136 (136 aa), were not recognized by some of the *S. enteritidis* sera, suggesting that the failure probably was due to the deletion of some dominant epitopes in the C-terminus between aa 151 and aa 165.

Of the four fragments containing the C-terminus of SEF14, two fragments, C145 (145 aa; aa 121-165) and C128 (128 aa; aa 38-165), reacted with all *S. enteritidis* sera. The other two fragments, C95 (95 aa) and C88 (88 aa), were not detected by some of the *S. enteritidis* sera, which suggested that the epitopes between aa 37 and aa 70 are associated with antibody binding. The amino acid sequence of the C128 fragment is provided in Figure 2B and is identified herein as SEQ ID NO. 3.

The hydrophilicity and antigenicity of the SEF14 amino acid residues were analyzed by the Hydrophilicity Plot : Kyte-Doolittle and Antigenic Index : Jameson-Wolf computer based programs. These programs assist in the prediction of the characteristics of the protein from the knowledge of the sequence. The regions of amino acids 41-53, 144-153 and 159-165 are hydrophilic, and it is believed that they represent antigenic epitopes. The deletion of these regions from SEF14 results in a polypeptide with decreased antigenicity.

The specificity of the SEF sub-fragments also was tested with sera from chickens experimentally infected with various *Salmonella* serotypes. Sub-fragment C128 did not react with sera from any *Salmonella* serotype other than *S. enteritidis*. Subfragment C145 reacted weakly with sera from infection of *S. senftenberg, S. java, S. pullorum, S. amsterdam* and *S. paratyphimurium*. The deletion of N-terminus aa 1-36 of F165 made subfragment C128 more specific than F165 or C145. When the F165 fragment was tested, cross-reactions could be observed, and slight cross-reactions also were observed when the C145 fragment was tested. In contrast, when C128 was tested, no cross reaction was seen. These results indicate that the region bounded by aa 1-36 contains at least one epitope shared by different *Salmonella* serotypes. These studies are described in detail in Example 1, below. From the foregoing, therefore, a desirable fragment within the scope of this invention is one which consists essentially of an antigenic subfragment of the C145 fragment of SEF14 which specifically recognizes *S. enteritidis* antibodies in a biological sample obtained from poultry and distinguishes between *S. enteritidis* and other *Salmonella* species. Such subfragments include the C128 subfragment, which consists essentially of amino acids 37-165 of the SEF14 amino acid sequence, and further subfragments thereof which specifically recognize *S. enteritidis* antibodies in a biological sample obtained from poultry and distinguish between *S. enteritidis* and other *Salmonella* species. Also included within the scope of this invention are polypeptides which

correspond to, or are identical to, one of these subfragments of the C145 fragment but include a conservative amino acid substitution for at least one amino acid in the sequence of the subfragment of the C145 fragment, provided that with said substitution(s) the sequence specifically recognizes *S. enteritidis* antibodies in a biological sample obtained from poultry and distinguishes between *S. enteritidis* and other *Salmonella* species.

The specificity and sensitivity of subfragment C128 also was studied using ELISA with sera obtained from guinea pigs hyperimmunized with *Salmonella spp.* and *Enterobactericae spp.* and sera from chickens experimentally infected with *Salmonella spp.* With sera from guinea pigs, the OD value for *S. enteritidis* was at least 2 times as high as those of other sera with the exception of *S. dublin*, which had a similar OD value due to cross-reaction of SEF14 fimbriae by *S. dublin*. This cross-reactivity with *S. dublin* is not a problem in terms of being able to use C128 as a diagnostic for the identification of poultry infected with *S. enteritidis* because *S. dublin* does not infect, and so cannot be isolated from, chickens. With sera obtained from chickens, all sera from chickens infected with *S. enteritidis* showed an OD value at least two times higher than those obtained from sera from chickens infected with other *Salmonella* serotypes. These data indicate that the reactivity of C128 in ELISA was the same as the reactivity in immunoblot. The cut-off OD value for positive reaction was three times that of the average OD of normal sera.

The C128 subfragment of F165 thus is a useful tool for the detection of *S. enteritidis* in poultry.

B. Antigenic fragment of flagellin antigen

The amino acid sequence of the flagellin region of *S. enteritidis* (nucleotides 754-1024 of *S. enteritidis* strain designated 13076 and deposited with and available from the ATCC as accession number U12963) is shown in Figure 5, identified as SEQ ID NO:4. A DNA fragment encoding the flagellin region was amplified and cloned into an expression vector. The vector was chosen such that the desired flagellin gene product would be expressed as a fusion protein. A preferred fusion partner is glutathione -S- transferase of *Schistosoma japonica* (GST). The resultant GST-flagellin protein recombinant protein was expressed. The amino acid sequence of the 90 amino acid fragment of the flagellin protein is provided in Figure 5. The sequence is identified herein as SEQ ID NO:5.

This recombinant protein was recognized by *Salmonella dublin* as well as by *S. enteritidis*, but not by any of the other *Salmonella spp.* tested.

To determine if a smaller sequence could be used to specifically identify *S. enteritidis* in poultry samples, the sequence of the flagellin portion of the recombinant protein was compared to the sequences of the flagellin domains of other *Salmonella spp.* and the specific regions within the protein that showed the most variation from the other sequences were identified. Four subfragments of the flagellin domain were expressed as fusion proteins ranging in size from 27.2kD to 31.6 kD, and the reactivity of each fragment

was tested. Each fragment was shown to specifically detect and discriminate samples from poultry infected with *S. enteritidis* from uninfected samples. These four subfragments are shown in Figure 5 and identified herein as SEQ ID NOS: 6-9.

The 90 amino acid fragment and each of the four subfragments illustrated in Figure 5 thus are useful in the method of the present invention. Also useful is an antigenic fragment of any of these sequences or an amino acid sequence which corresponds to one of these sequences which comprises a conservative amino acid substitution for at least one amino acid in the sequence, provided that with said substitution(s) the sequence specifically recognizes *S. enteritidis* antibodies in a biological sample obtained from poultry and distinguishes between *S. enteritidis* and other *Salmonella* species.

Detection of *S. enteritidis* infections

The antigenic fragments of *S. enteritidis* fimbrial and flagellin proteins can be used to detect *S. enteritidis* infections in samples obtained from poultry. Preferred samples are sera samples or yolk samples from poultry eggs. A sample is contacted with an antigenic fragment of *S. enteritidis* fimbrial or flagellin proteins in accordance with the present invention for a time and under conditions sufficient for the formation of an immunological complex between *S. enteritidis* antibodies present in the sample and the antigenic fragment. The formation of the resulting complex can be assayed either by direct detection

methods or indirect detection methods. Assays can be conducted in accordance with standard techniques. Depending upon the assay technique chosen, the antigenic fragment, *S. enteritidis* antibodies present in the sample, or secondary antibodies, if used, can be labeled with a detectable label. Suitable detectable labels can be chosen from fluorescent compounds, radioactive elements, enzymes capable of producing a reaction detectable compound, or gold.

Using direct detection methods, the antigen-antibody complexes can be assayed, for example by ELISA, in accordance with standard techniques. The direct detection assays can use labeled antigenic fragment or labeled anti-*S. enteritidis* antibodies. Labeling of the antibody or antigenic fragment can be conducted using standard labeling techniques. The detectable label can be a fluorescent compound, a radioactive element, an enzyme capable of producing a detectable reaction product, or gold. The selected antigenic fragment of *S. enteritidis* fimbrial or flagellin proteins can be labeled, for example, with a radioactive isotope. The sample then is contacted with a radioactively labeled antigen fragment of *S. enteritidis* fimbrial or flagellin proteins in accordance with the present invention for a time and under conditions sufficient for the formation of an immunological complex between *S. enteritidis* antibodies present in the sample and the antigenic fragment, such that antibody-antigen complexes which form are labeled with the radioactive label. The formed complex then can be assayed by immunoblotting. Alternatively, *S.*

enteritidis antibodies present in the sample can be labeled with a directly detectable label, such as a fluorescent compound or gold, or can be conjugated to an enzyme commonly used for colorimetric or fluorescent detection, such as alkaline phosphatase. Unlabeled antigenic fragment of *S. enteritidis* fimbrial or flagellin proteins then can be coated onto the microtiter plates and contacted with the biological sample containing labeled *S. enteritidis* antibodies so that, again, antibody-antigen complexes which form are labeled with the detectable label. Detection of the formed antigen-antibody complexes that are labeled can be conducted, for example, by a standard ELISA assay or other sensitive detection system.

Using indirect detection methods, the antigen-antibody complexes can be assayed, for example, by ELISA or immunoblotting in accordance with standard techniques. Antigenic fragments of *S. enteritidis* fimbriae or flagellin proteins can be coated onto microtiter plates which can provide a solid phase for capturing anti-*S. enteritidis* antibodies present in the sample. Alternatively, antigenic fragments can be provided in solution for immunoblotting. The fragment is contacted with the biological sample under conditions sufficient for the formation of an immunological complex between *S. enteritidis* antibodies present in the sample and the antigenic fragment. Antibody-antigen complexes that form then can be detected using secondary antisera conjugated to detectable labels, such as fluorescent-labeled antibodies, enzyme-conjugated antibodies, including

horseradish peroxidase-conjugated antibodies or alkaline phosphatase-conjugated antibodies, radioactive tracer-labeled antibodies or gold-labeled antibodies.

In an alternative to these assay techniques, monoclonal antibodies to the antigenic fragments of *S. enteritidis* of the present invention can be generated in accordance with standard techniques. The monoclonal antibodies then can be used to coat the wells of microtiter plates which then are contacted with a biological sample suspected of containing *S. enteritidis* and antigens in the sample will bind to the monoclonal antibodies and can be detected using a detectable label as described above.

A preferred assay for use in the present invention is an ELISA as described above. A second preferred assay is a lateral flow format assay, which can easily be provided in the form of a rapid test kit. Such kits, which are commercially available for other purposes, allow the absorption of fluids via application to a pre-determined well containing a strip of antigen, or as in this case, an antigenic fragment, on a membrane and then inserting the well into a device which has a window above the antigen-coated membrane. The sample is added to the well such that it flows across the membrane, allowing any antibodies present in the sample to interact with the antigen in the membrane. A secondary antibody which is labeled with a detectable label, such as gold, also is incorporated into the membrane and, upon wetting of the membrane with the sample, is mobilized and binds to any primary antibody present in the sample. Any positive result

obtained is observed by the appearance of a band on the membrane produced by the precipitation of the gold onto the membrane which is visualized through the window. The lateral flow format assay also can be modified by coating monoclonal antibodies generated against the desired antigenic fragment onto the membrane.

Western blotting, dot blots and quartz crystal technology also can be utilized for the purpose of detection of the antigen or monoclonal antibody.

Assays using the C128 fimbrial fragment and assays using one the flagellin fragments can be used independently for the detection of *S. enteritidis* infections in poultry and their eggs. It also can be useful to use the fimbriae and flagellin fragments in combination. Detectable antibodies against SEF14 indicate an early stage infection, while antibodies to the flagellin proteins typically are detected later in the infection, as the antibody to flagella is generated later but has a longer existence. Thus, by testing a sample with antigenic fragments of each of the fimbriae protein and flagellin protein, one can detect the presence of an infection from its beginning stages to its mature stages and thus ensure that no infections go undetected.

Diagnostic Kits

The invention further comprises diagnostic kits which can be used to detect and discriminate *S. enteritidis* infections in samples from poultry. The kits comprise an antigenic fragment of the *S. enteritidis* fimbrial protein or flagellin domain, as

described above, or can comprise both an antigenic fragment of the *S. enteritidis* fimbriae protein and an antigenic fragment of the *S. enteritidis* flagellin domain. The kit further can comprise a label. Suitable labels include labels which can be attached to the antigenic fragment or to antibodies present in the biological sample to be tested, such as an enzyme, gold, fluorescent compound, or radioactive element. Alternatively, the label can be provided in the form of a labeled secondary antibody for use in an indirect detection method, such as enzyme-conjugated antibodies, including horseradish peroxidase conjugated antibodies or alkaline phosphatase labeled antibodies, gold-labeled antibodies, fluorescently-labeled antibodies, or radiotracer-labeled antibodies.

In one preferred embodiment, the kit comprises the essential elements for a lateral flow format assay as described above.

The invention is further described in the following examples, which are provided for illustrative purposes and are not intended to be construed as limiting.

Example 1

Bacterial strains

S. enteritidis strains 2/93 phage type 4, 119/95 phage type 4, 330/96 phage type 11a and 131/97 phage type 37, 40/97 phage type 1, and 296/96 phage type 9b were provided by the Primary Production Department,

Singapore, and isolate 94/6510 was provided by the U.S. Department of Agriculture. All of these strains are publicly available. These strains were used for the experimental infection of chicken.

Serum samples

Sera Group 1

Guinea pigs were inoculated with different strains of bacterium to obtain serum specific to the following: *S. enteritidis* (group D), *S. dublin* (group C), *S. kentucky* (group C3), *S. heidelberg* (group B), *S. newport* (group C3), *S. typhimurium copenhagen* (group B), *S. cholersasius*, *S. anatum* (group E1), *S. cerro* (group K), *Escherichia coli*, *Aeromonas hydrophilus*, *Pasteurella multocida*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Yersinia enterococci*, *Citrofreundii*, *shigella sonnei*, *Serratia marscens* and uninfected guinea pig serum.

Sera group 2

Chickens were infected with various serotypes of *Salmonella* obtained from the Singapore General Hospital: *S. dublin* (group D), *S. typhimurium* (group B), *S. anatum* (group E1), *S. pullorum* (group D), *S. java* (group B), *S. paratyphi A* (group A), *S. haardt* (group C3), *S. hadar* (group C2), and *E. coli*.

Sera group 3

The six *S. enteritidis* strains listed above were prepared as an overnight broth which was diluted to 1 x 10^8 colony forming units (cfu). This broth in turn was diluted further, resulting in 10^7 and 10^5 concentrations of the six cultures. Twelve ten-week old chickens were

divided into six groups of two, and one chicken in each group was inoculated with 1 ml of the 10^5 bacterial broth and the second in each group inoculated with 1 ml of the 10^7 bacterial broth. Sera from each chicken was obtained at seven day intervals and the flock was monitored for *Salmonella* shedding from pre-inoculation to two weeks post-inoculation.

Sera group 4

Forty samples of *S. enteritidis* negative sera were obtained from specific pathogen-free chickens ranging in age from 1 day old chicks to ten week old chickens. Specifically, the forty samples comprised sera obtained from ten one day-old chicks, fourteen four week old chicks, and sixteen ten week old chicks.

Sera group 5

Twenty five serum samples were collected from chickens in the field by the Primary Production Department (PPD), Singapore. These samples were collected from farm chickens in Malaysia in which *S. enteritidis* infections had been identified.

Recombinant *S. enteritidis* antigen

The nucleotide sequence of the *sefA* gene is available through Genbank (accession number L03833). Oligonucleotide primers for the C128 fragment of the fimbrial SEF14 antigen were designed and synthesized as follows. Primers were designed with a Bam H1 restriction site in the 5' end for the forward primer and an EcoR1 restriction site at the 3' end for the reverse primer.

forward primer:

5' TGC AGC TCA GAA TAC AAC ATC A 3' (starting from base 112)

reverse primer:

5' GTT TTG ATA CTG CTG AAC GTA (end at base 495)

The forward and reverse primers are identified herein as SEQ ID NOS: 10 and 11, respectively.

The Beckman OLIGO 1000M DNA synthesizer was used in the synthesis. Using genomic DNA extracted from *S. enteritidis* strain 13076 ATCC, DNA fragments were amplified by PCR. The amplified DNA was cloned into pGEX-4T-3 expression vector (available from Pharmacia Biotech, Uppsala, Sweden). Clones containing inserts were sequenced to ensure the correctness of the reading frame. Proteins fused to GST were expressed in *E. coli* strain JM105, obtained from Amersham Pharmacia Biotech, Uppsala, Sweden; catalog no. 27-1550-01. For the purification of the fusion protein, the bacterial cell pellet was subjected to GST affinity column purification (Pharmacia Biotech), following the instructions provided by the manufacturer. To attain higher purity, the protein was loaded onto SDS-PAGE gel and the protein band visualized by a 1 minute stain with Coomassie brilliant blue and de-stained in di-ionized water. The band was excised and eluted in de-ionized water. The protein obtained through such gel purification was used as antigen in an ELISA test.

Immunoblot

Western blot analysis was carried out according to conventional format with some modifications. Purified fusion protein was mixed with sample buffer containing

0.1 mol/L DTT in final concentration. The mixture was denatured at 100°C for 3 minutes and separated by SDS-PAGE. The separated protein on gel was transferred to a nitrocellulose membrane by overnight electrophoresis under 20 volts. The membrane was blocked with 5% skim milk and stripped.

Serum samples were diluted at 1:400, added to each of the strips individually, and incubated at room temperature for 1 hour, followed by the incubation of rabbit anti-chicken IgG peroxidase conjugate for one hour and then by color development in 3'3'-diaminobenzidine tetrahydrochloride (DAB) substrate. All fimbrial fragments were found to be positive in reactivity.

ELISA

An ELISA was preformed using a conventional format. Purified protein was coated on 96-well flat bottom plates (NUNC) in carbonate buffer, pH 9.6 at 50 ng/100 µl per well. After blocking with 1% BSA, serum samples, diluted 1:200, were added and incubated at 37°C for 15 minutes, followed by incubation of secondary IgG peroxidase conjugate at 37°C for 15 minutes and finally by addition of substrate OPD. Results were expressed as the optical density (OD) at 492 nm by ELISA reader (Bio-dot).

The C128 antigen fragment was mapped to be specific and sensitive to *S. enteritidis* infection. the cutoff value for a positive reaction was 3 times the OD value of that of uninfected chicken sera. The fragment was tested against Group 2, where only *S.*

enteritidis was shown to be positive, Group 3, where all sera were shown to be positive in reactivity, and Group 4, where all sera were shown to be negative.

Example 2

The bacterial strains and the sera groups used were the same as in Example 1, with the exception that the chickens in Sera Group 2 were infected with fewer serotypes of *Salmonella*, as described below, than described in Example 1.

Recombinant *S. enteritidis* antigen

Nucleotides 754-1024 bp of the *S. enteritidis* DNA 13076 ATCC strain encoding the flagellar region were amplified through polymerase chain reaction (PCR) techniques. The amplified DNA was cloned into pGEX-2T expression vector (publicly available from Pharmacia Biotech, Uppsala, Sweden). For the purification of the GST-flagellin protein, the bacterial cell pellet was subjected to GST affinity column purification (Pharmacia Biotech) in accordance with the manufacturer's directions. Most of the GST-flagellin protein was recovered at this step. To attain higher purity, the protein was loaded onto a polyacrylamide gel. The band was excised and eluted in de-ionized water containing 0.1% SDS.

ELISA procedure

Immulon microtiter plates (available from Dynatech Laboratories Inc. Chantilly, Va.) were coated with 50 ng/100 microliters/well recombinant protein in 0.1M sodium bicarbonate buffer (ph 9.6). The plates were incubated at 37°C for 4 hours and then refrigerated until further use. The plates were washed 4 times with ELISA washing solution (phosphate buffered saline, 0.05% Tween 20[PBST]) and excess binding sites were saturated with 1% bovine serum albumin (BSA) fraction V (Sigma) in phosphate buffered saline (pH 7.4) for 1 hour at 37°C. After four washings, 100 microliters of the test serum sample(1:200 dilution) in 1% BSA-PBS buffer were added to each well, and the plates were incubated for 10 minutes at 37°C. Following subsequent washing of the wells, 100 microliters of anti-chicken immunoglobulins conjugated with horseradish peroxidase (KPL) were added to each well and the plates were incubated at 37°C for 10 minutes. The wells were washed again and 100 microliters of the substrate solution (2'2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) with H_2O_2) were added. The color reaction was allowed to proceed at room temperature for 10 minutes, and the absorbance of each well at 405 nm (ABTS) respectively was recorded in an automatic ELISA plate reader.

Mapping of the *S. enteritidis* partial protein

In order to assess if the obtained partial protein, shown in Figure 5 as SEQ. ID NO:5, was the smallest fragment usable for the purpose of detecting the presence of *S. enteritidis* in poultry or their

eggs, primers were designed to amplify specific regions within the partial fragment which showed the greatest sequence variation between *S. enteritidis* and other flagellin domains of *Salmonella* serotypes identified in Asten et al., *J. Bacteriology* 177(6):1610 (1995). Primers (set forth in the table below) and the resulting fragments were cloned using the same method set forth above in vector pGEX-2T. The four resulting partial protein fragments were designated FLP-A, FLP-B, FLP-C and FLP-D. These partial protein fragments also were purified by affinity chromatography and gel excision. These partial protein fragments were quantified and subsequently coated as 50 ng per well. Characterization of these partial protein fragments was conducted with the use of chicken sera in ELISA. Serum obtained from uninfected chickens yielded negative results, while serum obtained from chicken infected with *S. enteritidis* yielded positive results.

Table 1 Nucleotide sequences of primers used

Fragments	*Primers Used*	*Nucleotides amplified*
A: Forward A: Reverse	5'AAGGATCCAAAGGTGGTAAGGAAGGA5' 5'CCGAATTCTTTGTCACCGTTCACTAC3'	775bp to 982bp'
B: Forward B: Reverse	5'AAGGATCCAAAACTGGTGATGACGGT3' 5'CCGAATTCGCTTGATTGTAAGGTAGC3'	828bp TO 948bp
C: Forward C: Reverse	5'AAGGATCCGACGGTAATGGTAAGGTT3' 5'CCGAATTCCGTCGCGCCAGTGGCAAT3'	838bp to 919 bp

D: Forward E: Reverse	5'AAGGATCCTCTACTACCATCAATGGT3' 5'CCGAATTCTAACGTAACTTTTTCACC3'	856bp to 889 bp

The primers listed above are identified herein as SEQ ID NOS: 12-19, respectively.

Data analysis

The average reading was calculated for the negative sera used in these assays and the standard deviation calculated. A range of standard deviations were calculated, where the average of the negative sera was added to one, two, three, four or five standard deviations. It was found that the average of the negative reference sera plus two of their standard deviations was sufficient in value to include all negatives and all the known positive sera values obtained were above that value. The cut off value (detection limit) of the ELISA was defined as the mean value of the negative reference sera plus two times their standard deviation, which is able to discriminate the positive sera and also field infected sera.

The positive/negative ratio also was calculated and these figures serve as a relative indicator strength of the reaction over the negative reference values.

Differences in the sets of data collected were statistically analyzed by the t-test (Sigma Plot program). The value obtained indicates whether the differences seen in two sets are significantly different. P values of less than 0.01 are considered to be statistically different.

Characterization and specificity of the *S. enteritidis* flagellin fusion protein

Gel purified GST-*S. enteritidis* antigen was immunologically characterized by Western Blot. The fusion protein was tested with sera raised from guinea pigs from Group 1 sera. Of the ten *Salmonella* serotypes tested, only *S. enteritidis* and *S. dublin* of the same serogroup displayed reactivity. The sera raised from other serotypes and other organisms in Group 1 showed no reactivity. All sera were screened at a dilution of 1:50. The fusion protein then was screened against experimentally infected and non-infected sera obtained from chickens. Group 3 sera all reacted positively and Group 4 sera showed no reaction.

Development of ELISA for *S. enteritidis* using GST-*S. enteritidis* fusion protein

Gel purified GST-*S. enteritidis* flagellin antigen fragment fusion protein was diluted in ELISA coating buffer and used as coating antigen on microtiter plates at 50 ng per well overnight. The coated antigen was characterized by the guinea pig sera from Group 1. Subsequent characterization was carried out using chicken sera. Sera from *S. enteritidis*-infected chickens had the highest absorbance value, which was calculated to have a positive/negative ratio of 4.59 times greater than the average of the negative references.

Non-infected chicken sera from Group 3 (sera obtained as pre-bleeds from the chickens prior to infection) then were tested against the coated antigen. All yielded almost basal readings. Infected flock sera from Group 3 which were of 5 different phage types yielded 100% positive results. Thus, this test is able to detect *S. enteritidis* of different phage types.

Sera from Group 2, obtained from the flock experimentally infected with *S. haardt, S. typhimurium, S. hadar, S. typhi* and *S. java*, gave negative results with the test antigen. These data confirm that there are no cross-reactions with other *Salmonella* serotypes.

Comparison with commercially available IDEXX *Salmonella enteritidis* test kit

Group 5 sera were evaluated using both the IDEXX commercial test kit (IDEXX Laboratories Inc., Westbrook, ME) and the antigen of the present invention. Using the IDEXX kit, 3/28 sera were detected; using the antigen of the present invention, 14/28 samples were identified as serologically positive. The antigen of the present invention thus showed a detection rate which was 78.6% greater than obtained using the commercially available test kit.

ELISA results: mapped fragments

The *S. enteritidis* fusion protein was further mapped to assess the smallest region which could still detect and discriminate infected from non-infected sera. A total of four fragments, shown in Figure 5 as fragments A-D and having molecular weights (inclusive

of GST) 31.6kD, 30.4 kD, 29 kD, and 27.2 kD, were cloned and screened with the selected positive and negative sera. Eight serum samples from chicken experimentally infected with *S. enteritidis* and eight samples of SPF (specific pathogen free) sera were used to qualify each fragment for its sensitivity. Positive/negative (P/N) ratios for the fragments were determined. The average P/N ratio for each of the fragments was as follows:

fragment A: 3.23
fragment B: 2.95
fragment C: 3.02
fragment D: 2.97

These figures reflect a similar reactivity pattern to that of the original fusion antigen, which has a P/N ratio of 3.01.

The fragments were tested for specificity with other *Salmonella* serotypes present in the samples of serum in Group 2. None of the fragments reacted with any of the serotypes other than *S. enteritidis*. The results are shown in Table 2 below:

Table 2

Fragment Reactivity with Serotype Specific Serum

Fragments/ Serum	270	A	B	C	D
S.enteritidis	+	+	+	+	+
S.dublin	-	-	-	-	-
S.typhimurium	-	-	-	-	-

S.anatum	-	-	-	-	-
S.pullorum	-	-	-	-	-
S.java	-	-	-	-	-
S.paratyphi	-	-	-	-	-
S.haardt	-	-	-	-	-
S.hadar	-	-	-	-	-
E.coli	-	-	-	-	-

Claims:

1. A method for detecting *Salmonella enteritidis* in a biological sample obtained from poultry which comprises

contacting said biological sample with an antigenic fragment of *S. enteritidis* fimbrial protein or an antigenic fragment of *S. enteritidis* flagellin protein under conditions sufficient for the formation of an immunological complex between *S. enteritidis* antibodies present in said sample and said fragment, and

detecting the formation of such a complex; wherein said fragment specifically recognizes *S. enteritidis* antibodies present in the sample and discriminates between *S. enteritidis* and other *Salmonella spp*.

2. The method of claim 1, wherein said sample comprises sera or egg yolk.

3. The method of claim 1, wherein said sample is contacted with a fragment of *S. enteritidis* fimbrial protein.

4. The method of claim 3, wherein said fragment is provided as a fusion polypeptide wherein an additional polypeptide is fused to said fragment.

5. The method of claim 3, wherein said fragment consists essentially of a subfragment of amino acids 21-165 of SEQ ID NO:2, an antigenic portion thereof, or a sequence which corresponds to a subfragment of amino acids 21-165 of SEQ ID NO:2 comprising a conservative amino acid substitution for at least one amino acid in said sequence.

6. The method of claim 3, wherein said fragment consists essentially of amino acids 38-165 of SEQ ID NO:2, or an antigenic portion thereof, or a sequence which corresponds to said amino acids 38-165 of SEQ ID NO:2 and comprises a conservative amino acid substitution for at least one amino acid in said sequence.

7. The method of claim 1, wherein said sample is contacted with a fragment of *S. enteritidis* flagellin protein.

8. The method of claim 7, wherein said fragment is provided as a fusion polypeptide wherein an additional polypeptide is fused to said fragment.

9. The method of claim 7, wherein said fragment consists essentially of the amino acid sequence of SEQ ID NO:5, an antigenic fragment thereof, or a sequence which corresponds to said sequence and comprises a conservative amino acid substitution for at least one amino acid in said sequence.

10. The method of claim 7, wherein said fragment consists essentially of the amino acid sequence of SEQ ID NO:6, an antigenic fragment thereof or a sequence which corresponds to said sequence which comprises a conservative amino acid substitution for at least one amino acid in said sequence.

11. The method of claim 7, wherein said fragment consists essentially of the amino acid sequence of SEQ ID NO:7, an antigenic fragment thereof or a sequence which corresponds to said sequence which comprises a conservative amino acid substitution for at least one amino acid in said sequence.

12. The method of claim 7, wherein said fragment consists essentially of the amino acid sequence of SEQ ID NO:8, an antigenic fragment thereof or a sequence which corresponds to said sequence which comprises a conservative amino acid substitution for at least one amino acid in said sequence.

13. The method of claim 7, wherein said fragment consists essentially of the amino acid sequence of SEQ ID NO:9, an antigenic fragment thereof or a sequence which corresponds to said sequence which comprises a conservative amino acid substitution for at least one amino acid in said sequence.

14. The method of claim 1, wherein said fragment is labeled with a detectable label.

15. The method of claim 14, wherein said label comprises a fluorescent compound, a radioactive element, an enzyme capable of producing a reaction detectable compound or gold.

16. The method of claim 1, wherein said sample has been contacted with a detectable label which binds to anti-*S. enteritidis* antibodies present in said sample.

17. The method of claim 16, wherein said label comprises a fluorescent compound, a radioactive element, an enzyme capable of producing a reaction detectable compound or gold.

18. An isolated fragment of *S. enteritidis* fimbrial protein consisting of the amino acid sequence of SEQ ID NO:3, an antigenic fragment thereof, or a sequence which corresponds to said sequence which comprises a conservative amino acid substitution for at least one amino acid in said sequence.

19. An isolated fragment of *S. enteritidis* flagellin protein consisting of the amino acid sequence of SEQ ID NO:6, an antigenic fragment thereof, or a sequence which corresponds to said sequence which comprises a conservative amino acid substitution for at least one amino acid in said sequence.

20. An isolated fragment of *S. enteritidis* flagellin protein consisting of the amino acid sequence

of SEQ ID NO:7, an antigenic fragment thereof or a sequence which corresponds to said sequence which comprises a conservative amino acid substitution for at least one amino acid in said sequence.

21. An isolated fragment of *S. enteritidis* flagellin protein consisting of the amino acid sequence of SEQ ID NO:8, an antigenic fragment thereof, or a sequence which corresponds to said sequence and comprises a conservative amino acid substitution for at least one amino acid in said sequence.

22. An isolated fragment of *S. enteritidis* flagellin protein consisting of the amino acid sequence of SEQ ID NO:9, an antigenic fragment thereof or a sequence which corresponds to said sequence and comprises a conservative amino acid substitution for at least one amino acid in said sequence.

23. A kit comprising (a) a fragment of *S. enteritidis* fimbrial or flagellin protein which specifically recognizes *S. enteritidis* antibodies present in a biological sample obtained from poultry suspected of being infected with *S. enteritidis* and discriminates between antibodies from *S. enteritidis* and other *Salmonella spp.* and (b) a detectable label.

24. A kit comprising a fragment of *S. enteritidis* fimbrial protein and a fragment of *S. enteritidis* flagellin protein, each of which fragments specifically recognizes *S. enteritidis* antibodies present in a

biological sample obtained from poultry suspected of being infected with *S. enteritidis* and discriminates between antibodies from *S. enteritidis* and other *Salmonella spp*..

25. A kit in accordance with claim 24, which further comprises a detectable label.

26. A method for detecting *Salmonella enteritidis* in a biological sample obtained from poultry which comprises

contacting said biological sample with an antigenic fragment of *S. enteritidis* fimbrial protein and an antigenic fragment of *S. enteritidis* flagellin protein under conditions sufficient for the formation of an immunological complex between *S. enteritidis* antibodies present in said sample and either or both of said fragments, and

detecting the formation of such a complex or complexes;

wherein each of said fragments specifically recognizes *S. enteritidis* antibodies present in the sample and discriminates between *S. enteritidis* and other *Salmonella spp*.

27. A method for detecting *Salmonella enteritidis* in a biological sample obtained from poultry which comprises

contacting a first portion of said biological sample with an antigenic fragment of *S. enteritidis* fimbrial protein under conditions sufficient for the

formation of an immunological complex between *S. enteritidis* antibodies present in said sample and said fragment; and

detecting the formation of such a complex; wherein said fragment specifically recognizes *S. enteritidis* antibodies present in the sample and discriminates between *S. enteritidis* and other *Salmonella spp.*; and

contacting a second portion of said biological sample with an antigenic fragment of *S. enteritidis* flagella protein under conditions sufficient for the formation of an immunological complex between *S. enteritidis* antibodies present in said sample and said fragment, and

detecting the formation of such a complex; wherein said fragment specifically recognizes *S. enteritidis* antibodies present in the sample and discriminates between *S. enteritidis* and other *Salmonella spp.*

28. The method of claim 26 or 27, wherein said sample comprises sera or egg yolk.

29. The method of claim 26 or 27, wherein each of said fragments is provided as a fusion polypeptide wherein an additional polypeptide is fused to said fragment.

30. The method of claim 26 or 27, wherein said fragment of *S.enteritidis* fimbrial protein consists essentially of a subfragment of aa 121-165 of the amino

acid sequence of SEQ ID NO:2, an antigenic fragment thereof, or a sequence which corresponds to said sequence and comprises a conservative amino acid substitution for at least one amino acid in said sequence.

31. The method of claim 26 or 27, wherein said fragment of *S. enteritidis* fimbrial protein consists essentially of the amino acid sequence of SEQ ID No:3, an antigenic fragment thereof, or a sequence which corresponds to said sequence and comprises a conservative amino acid substitution for at least one amino acid in said sequence.

32. The method of claim 26 or 27, wherein said fragment of *S. enteritidis* flagella protein consists essentially of the amino acid sequence of SEQ ID NO:5, an antigenic fragment thereof or a sequence which corresponds to said sequence and comprises a conservative amino acid substitution for at least one amino acid in said sequence.

33. The method of claim 26 or 27, wherein each of said fragments is labeled with a detectable label.

34. The method of claim 26 or 27, wherein said label comprises a fluorescent compound, a radioactive element, an enzyme capable of producing a reaction detectable compound or gold.

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(54) Title: DETECTION OF *SALMONELLA ENTERITIDIS*

(57) Abstract: A method for detecting *Salmonella enteritidis* in poultry and their eggs comprises contacting a biological sample obtained from poultry suspected of containing *S. enteritidis* with a fragment of a *S. enteritidis* fimbrial protein or a fragment of a *S. enteritidis* flagellin protein which specifically recognizes *S. enteritidis* antibodies present in the sample and discriminates between *S. enteritidis* and other *Salmonella spp.*

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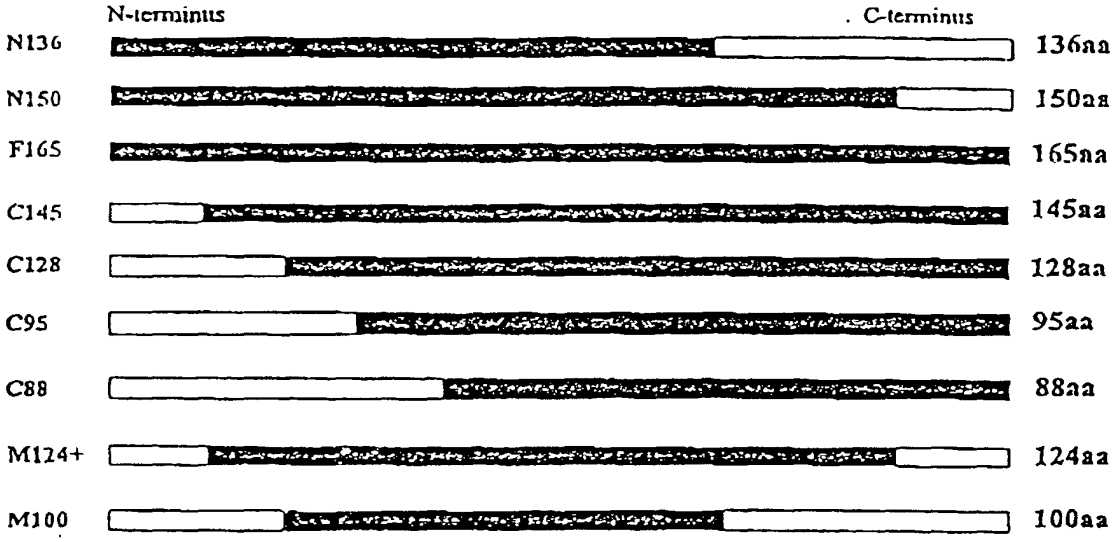
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C-terminus
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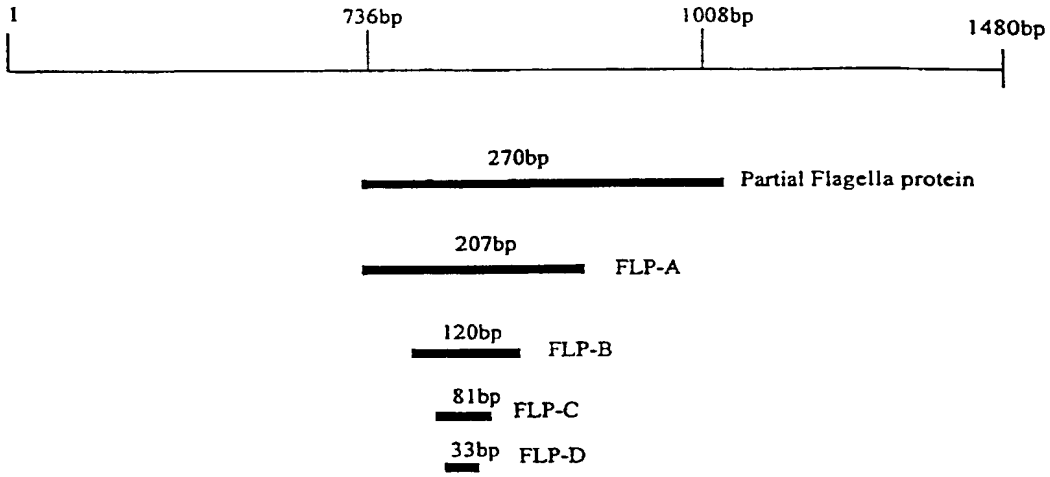
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270bp
Partial Flagella protein
207bp
FLP-A
120bp
FLP-B
81bp
FLP-C
33bp
FLP-D

DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)		
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	COMPLETE IF KNOWN	
	Application Number	10/018,892
	Filing Date	December 26, 2001
	Group Art Unit	
	Examiner Name	

As a below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: DETECTION OF SALMONELLA ENTERITIDIS the specification of which was filed on June 22, 1999 as PCT International Application Number PCT/SG99/00061.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Numbers	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			☐ ☐	☐ ☐	☐ ☐

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)

I or we hereby appoint the registered practitioner(s) associated with Customer No. 6449 to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith. Direct all correspondence to Customer Number 6449.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF SOLE OR FIRST INVENTOR:	[ ] A petition has been filed for this unsigned inventor		
Given Name (first and middle [if any]) 1-00 Hwei-Sing		Family Name or Surname KWANG	
Inventor's Signature		Date 25/6/02	
Residence: City Singapore SGX		Country Singapore	Citizenship USA
Mailing Address 54 West Coast Crescent, #03-02, West Bay Condimin.			
Mailing Address			
City Singapore		Postal Code 428037	Country Singapore
NAME OF SECOND INVENTOR:	[ ] A petition has been filed for this unsigned inventor		
2-00 Given Name (first and middle [if any]) Wei		Family Name or Surname LIU	
Inventor's Signature		Date 25-06-2002	
Residence: City Singapore SGX		Country Singapore	Citizenship China
Mailing Address ~~Block 508, West Coast Drive, #08-265~~ BIK 280 Toh Guan Rd #12-205 Singapore 600280			
Mailing Address			
City Singapore		Postal Code 120508	Country Singapore
NAME OF THIRD INVENTOR:	[ ] A petition has been filed for this unsigned inventor		
3-00 Given Name (first and middle [if any]) Su-Shing Sharon		Family Name LOW or Surname	
Inventor's Signature		Date	
Residence: City Singapore SGX		Country Singapore	Citizenship Singapore
Mailing Address 17C Nassim Road, #01-04 Nassim Park			
Mailing Address			
City Singapore		Postal Code 258394	Country Singapore
NAME OF FOURTH INVENTOR:	[ ] A petition has been filed for this unsigned inventor		
4-00 Given Name (first and middle [if any]) Hilda Kwanyeng		Family Name LOH or Surname	
Inventor's Signature		Date	
Residence: City Singapore SGX		Country Singapore	Citizenship Singapore
Mailing Address 73, Cavanaugh Road, #08-372			
Mailing Address			
City Singapore		Postal Code 229624	Country Singapore

NAME OF SOLE OR FIRST INVENTOR:	[ ] A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any]) Hwei-Sing	Family Name or Surname KWANG	
Inventor's Signature	Date	
Residence: City Singapore	Country Singapore	Citizenship USA
Mailing Address 54 West Coast Crescent, #03-02, West Bay Condimin.		
Mailing Address		
City Singapore	Postal Code 426037	Country Singapore

NAME OF SECOND INVENTOR:	[ ] A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any]) Wei	Family Name or Surname LIU	
Inventor's Signature	Date	
Residence: City Singapore	Country Singapore	Citizenship China
Mailing Address Block 508, West Coast Drive, #08-285		
Mailing Address		
City Singapore	Postal Code 120508	Country Singapore

NAME OF THIRD INVENTOR:	[ ] A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any]) Su-Shing Sharon	Family Name LOW or Surname	
Inventor's Signature	Date	
Residence: City Singapore	Country Singapore	Citizenship Singapore
Mailing Address 17C Nassim Road, #01-04 Nassim Park		
Mailing Address		
City Singapore	Postal Code 258394	Country Singapore

NAME OF FOURTH INVENTOR:	[ ] A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any]) Hilda Kwanyeng	Family Name LOH or Surname	
Inventor's Signature	Date 18-6-2002	
Residence: City Singapore	Country Singapore	Citizenship Singapore
Mailing Address 73, Cavanagh Road, #08-372		
Mailing Address		
City Singapore	Postal Code 229624	Country Singapore

DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

☐ Declaration Submitted with Initial Filing

☒ Declaration Submitted after Initial Filing

Attorney Docket No.	2577-112
First Named Inventor	Hwei-Sing Kwang
COMPLETE IF KNOWN	
Application Number	10/018,892
Filing Date	December 26, 2001
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

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I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: DETECTION OF SALMONELLA ENTERITIDIS the specification of which was filed on June 22, 1999 as PCT International Application Number PCT/SG99/00061.

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Prior Foreign Application Numbers	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			☐	☐	☐

I hereby claim the benefit under 36 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)

I or we hereby appoint the registered practitioner(s) associated with Customer No. 6449 to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith. Direct all correspondence to Customer Number 6449.

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Inventor's Signature	Date	
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Mailing Address		
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Inventor's Signature	Date	
Residence: City Singapore	Country Singapore	Citizenship China
Mailing Address Block 508, West Coast Drive, #08-286		
Mailing Address		
City Singapore	Postal Code 120508	Country Singapore

NAME OF THIRD INVENTOR:	[ ] A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any]) Su-Shing Sharon	Family Name LOW or Surname	
Inventor's Signature	Date 9th July 2002	
Residence: City Singapore	Country Singapore	Citizenship Singapore
Mailing Address 17C Nassim Road, #01-04 Nassim Park		
Mailing Address		
City Singapore	Postal Code 258394	Country Singapore

NAME OF FOURTH INVENTOR:	[ ] A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any]) Hilda Kwanyeng	Family Name LOH or Surname	
Inventor's Signature	Date	
Residence: City Singapore	Country Singapore	Citizenship Singapore
Mailing Address 73, Cavanaugh Road, #08-372		
Mailing Address		
City Singapore	Postal Code 229624	Country Singapore

SEQUENCE LISTING

<110> Kwang, Hwei-Sing
Liu, Wei
Low, Su-Shing Sharon
Loh, Hilda Kwanyeng

<120> Detection of Salmonella Enteritidis

<130> 2577-112

<150> PCT/SG99/00061
<151> 1999-06-22

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tgttgctgct ggtcagaaag ttggtactct cagcattact gctactggtc cacataactc 240

agtatctatt gcaggtaaag gggcttcggt atctggtggt gtagccactg tcccgttcgt 300

tgatggacaa ggacagcctg ttttccgtgg gcgtattcag ggagccaata ttaatgacca 360

agcaaatact ggaattgacg ggcttgcagg ttggcgagtt gccagctctc aagaaacgct 420

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35 40 45

Gln Asp Pro Gly Phe Thr Gly Pro Ala Val Ala Ala Gly Gln Lys Val
50 55 60

Gly Thr Leu Ser Ile Thr Ala Thr Gly Pro His Asn Ser Val Ser Ile
65 70 75 80

Ala Gly Lys Gly Ala Ser Val Ser Gly Gly Val Ala Thr Val Pro Phe
85 90 95

Val Asp Gly Gln Gly Gln Pro Val Phe Arg Gly Arg Ile Gln Gly Ala
100 105 110

Asn Ile Asn Asp Gln Ala Asn Thr Gly Ile Asp Gly Leu Ala Gly Trp
115 120 125

Arg Val Ala Ser Ser Gln Glu Thr Leu Asn Val Pro Val Thr Thr Phe
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20 25 30

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35 40 45

Ser Val Ser Gly Gly Val Ala Thr Val Pro Phe Val Asp Gly Gln Gly
50 55 60

Gln Pro Val Phe Arg Gly Arg Ile Gln Gly Ala Asn Ile Asn Asp Gln
65 70 75 80

Ala Asn Thr Gly Ile Asp Gly Leu Ala Gly Trp Arg Val Ala Ser Ser
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35 40 45

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50 55 60

Thr Thr Glu Gly Ala Leu Asn Glu Ile Asn Asn Asn Leu Gln Arg Val
65 70 75 80

Arg Glu Leu Ser Val Gln Ala Thr Asn Gly Thr Asn Ser Asp Ser Asp
85 90 95

Leu Lys Ser Ile Gln Asp Glu Ile Gln Gln Arg Leu Glu Glu Ile Asp

100 105 110

Arg Val Ser Asn Gln Thr Gln Phe Asn Gly Val Lys Val Leu Ser Gln
115 120 125

Asp Asn Gln Met Lys Ile Gln Val Gly Ala Asn Asp Gly Glu Thr Ile
130 135 140

Thr Ile Asp Leu Gln Lys Ile Asp Val Lys Ser Leu Gly Leu Asp Gly
145 150 155 160

Phe Asn Val Asn Gly Pro Lys Glu Ala Thr Val Gly Asp Leu Lys Ser
165 170 175

Ser Phe Lys Asn Val Thr Gly Tyr Asp Thr Tyr Ala Ala Gly Ala Asp
180 185 190

Lys Tyr Arg Val Asp Ile Asn Ser Gly Ala Val Val Thr Asp Ala Ala
195 200 205

Ala Pro Asp Lys Val Tyr Val Asn Ala Ala Asn Gly Gln Leu Thr Thr
210 215 220

Asp Asp Ala Glu Asn Asn Thr Ala Val Asp Leu Phe Lys Thr Thr Lys
225 230 235 240

Ser Thr Ala Gly Thr Ala Glu Ala Lys Ala Ile Arg Gly Ala Ile Lys
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Gly Gly Lys Glu Gly Asp Thr Phe Asp Tyr Lys Gly Val Thr Phe Thr
260 265 270

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275 280 285

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305 310 315 320

Thr Ser Val Val Asn Gly Gln Phe Thr Phe Asp Asp Lys Thr Lys Asn
325 330 335

Glu Ser Ala Lys Leu Ser Asp Leu Glu Ala Asn Asn Ala Val Lys Gly
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Glu Ser Lys Ile Thr Val Asn Gly Ala Glu Tyr Thr Ala Asn Ala Thr
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Gly Asp Lys Ile Thr Leu Ala Gly Lys Thr Met Phe Ile Asp Lys Thr
370 375 380

Ala Ser Gly Val Ser Thr Leu Ile Asn Glu Asp Ala Ala Ala Ala Lys
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Val Asp Ala Val Arg Ser Ser Leu Gly Ala Ile Gln Asn Arg Phe Asp
420 425 430

Ser Ala Ile Thr Asn Leu Gly Asn Thr Val Thr Asn Leu Asn Ser Ala
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Arg Ser Arg Ile Glu Asp Ala Asp Tyr Ala Thr Glu Val Ser Asn Met
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Val Val Asn Gly Gln
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